Transcription factor IIA of wheat and human function similarly with plant and animal viral promoters

Christine Burke[1], Xu-Bo Yu[+], Louis Marchitelli[§], Elizabeth A.Davis and Steven Ackerman*
Biology Department, University of Massachusetts, Boston, MA 02125, USA

Received January 22, 1990; Revised and Accepted May 8, 1990

ABSTRACT

Eucaryotic transcription initiation by RNA polymerase II involves protein:DNA interactions during the formation of a transcription complex. In addition to RNA polymerase II there are at least five other general transcription factors necessary for initiation with the adenovirus major late promoter. One of these, TFIIA, is involved in the earliest events during transcription complex assembly. We have purified TFIIA from wheat germ and characterized it in an *in vitro* transcription system. Wheat TFIIA is a single polypeptide of M_r ~35 kd which functionally replaces human (HeLa) TFIIA to form a wheat/HeLa transcription system. [This polypeptide can be eluted from a SDS-polyacrylamide gel, refolded to a native conformation, and will function as wheat TFIIA in the heterologous system.] The heterologous system requires a lower optimal incubation temperature than the HeLa system. Biochemical characterization, using the adenovirus major late promoter, indicates that transcription reaction parameters for both wheat and HeLa TFIIA are similar but the kinetics of transcription for both TFIIAs are somewhat dissimilar. A plant viral promoter, the cauliflower mosaic virus 35S promoter, accurately and efficiently directs *in vitro* transcription in both the wheat/HeLa and HeLa systems with identical transcription kinetics. We conclude that TFIIA function has been conserved during evolution.

INTRODUCTION

Synthesis of eucaryotic pre-mRNAs is a multi-step process involving numerous protein transcription factors (TFs) and RNA polymerase II (reviewed in 1–3). Reconstituted *in vitro* transcription systems using purified proteins and/or altered templates have facilitated delineation of the steps in assembly of DNA:protein transcription complexes. These steps include interactions between proteins, proteins and DNA, and also conformational transitions (4–11). The observations that TFs from widely divergent species are functionally similar in heterologous systems (12–16) suggest that the basic mechanisms affecting transcriptional gene regulation may be common to all eucaryotes. Furthermore, enzymic analyses of RNA polymerase II from plants and animals demonstrates functional similarity (reviewed in 17).

The adenovirus-2 major late promoter (Ad-2 MLP) provides a minimal promoter model. Only sequences from the TATA box to the transcription start site are absolutely required (18) for transcription although upstream sequences (such as the MLTF region) may increase efficiency. Using the Ad-2 MLP at least five human (HeLa) general TFs (IIA, IIB, IID, IIE, and IIF) have been shown to be necessary for transcription initiation; these are also required by other class II genes (8,9,18–22). We have been interested in TFIIA because it is the first TF to interact with the DNA (5,8,11,21,23–25) and may also have a role in catalysis (26). It has been proposed that TFIIA either prepares the TATA region of the promoter for binding of TFIID, or that its association with TFIID facilitates that binding (24,25,27). For some genes TFIIA may be replaced by other TFs (such as ATF) which has different sequence specificity but performs essentially the same function of directing TFIID to the TATA box (29).

Another interesting feature of TFIIA is its effect on RNA polymerase II; purified RNA polymerase II is not processive (28) but becomes more processive *in vitro* in the presence of TFIIA (26). TFIIA may, therefore, have two functions during transcription.

In vitro transcription studies using soluble plant protein extracts are not possible or are limited to analysis of specific genes (68). We observed that wheat germ nuclear protein extracts accurately initiate transcription by RNA polymerase II (12). Following fractionation of these extracts to remove inhibitors and to resolve individual TFs, we identified and partially purified a wheat protein which substituted for HeLa TFIIA, maintained accurate initiation in a HeLa transcription system (12,13), and made RNA polymerase II more processive (26). As a first approach to developing a plant *in vitro* transcription system we are sequentially replacing HeLa TFs with those from wheat. In this report we demonstrate the direct participation of wheat TFIIA in transcription, and biochemically characterize it in a wheat/HeLa system. Wheat TFIIA is a polypeptide of M_r ~35 kd which substitutes for HeLa TFIIA and exhibits similar transcription

* To whom correspondence should be addressed

Present addresses: [+]Immulogic Pharmaceutical Corp., One Kendall Square, Cambridge, MA 02139 and [§]Exercise Physiology Division, USARIEM, Kansas St Natick, MA 01760, USA

parameters except for a lower temperature optimum. [Other plant TFs that have been identified or purified have been characterized by their specificity for a regulatory DNA sequence, but no direct evidence for their participation in transcription has been presented due to the lack of a plant transcription system (eg. 30–33)]. We also show that a plant viral promoter, the cauliflower mosaic virus (CaMV) 35S promoter, accurately and efficiently directs transcription in both a HeLa system and a wheat/HeLa system with similar kinetics.

MATERIALS AND METHODS

All compounds were of ultrapure quality and were obtained as described previously (11,35). HeLa cell nuclear extracts and fractions C, D, DB, DE, F, and FS, as well as wheat germ nuclear extracts and fractions F, K, KD, and KB were prepared as described (refs. 12,13,35 and Fig. 1). Chromatographic work was performed at 3°C and all solutions were autoclaved when possible.

HeLa nuclear extracts, prepared from 2.4×10^{11} cells, were fractionated by chromatography on phosphocellulose (11,25). TFIIA was further purified as described (ref. 35 and Fig. 1). TFIID was further purified by diluting an aliquot of fraction D (4 ml) to 0.05 M KCl with buffer A (20 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 20% glycerol) and chromatographing it on DEAE-cellulose (1.6 ml). TFIID activity was eluted (0.4 ml fractions) with 0.4 M KCl-buffer A (fraction DB, 1.5 ml), had BSA added to 0.1 mg/ml, and was dialyzed (for 1 hr) against 0.1 M KCl-buffer B (buffer B is the same as buffer A except that it additionally contains 5 mM $MgCl_2$). When necessary fraction DB (0.75 ml) was diluted to 0.1 M KCl by addition of buffer A prior to chromatography on a single-stranded DNA-cellulose (Sigma) column (2 ml). TFIID activity eluted (0.3 ml fractions) with 0.5 M KCl-buffer A (fraction DE, 1.5 ml), had BSA added to 0.1 mg/ml, and was dialyzed against 0.1 M KCl-buffer B (for 1 hr).

Wheat fraction KB (TFIIA) was further purified using a Sephacryl-S200 column (1×100 cm) (Fig.1). To 12 ml of fraction KB $(NH_4)_2SO_4$ (0.45 g/ml) was added, the mixture stirred for 1 hr, precipitated proteins collected by centrifugation at 15,000 rpm for 1 hr, and resuspended in 0.75 ml of 0.3 M KCl-buffer B. One-third of the resuspended fraction was loaded onto the Sephacryl column and chromatographed with 0.3 M KCl-buffer B. (The remainder of the resuspended pellet was stored at −80°C.) Fractions (1 ml) were collected and BSA added (to 0.1 mg/ml) to fractions 30 to 70 to stabilize TFIIA activity. Relative protein content was determined using 5 µl aliquots in the Bio Rad protein micro-assay. Aliquots (75 µl) were dialyzed (using a microdialyzer and 0.1 M KCl-buffer B, for at least 1 hr) and then assayed in an *in vitro* transcription system depleted of HeLa TFIIA and containing HeLa fraction C and TFIID. Undialyzed fractions with transcription activity were combined (fraction KI) and dialyzed against 0.1 M KCl-buffer B. Attempts to purify wheat TFIIA without adding BSA to the fractions, even at just the final chromatography step, were unsuccessful because TFIIA rapidly lost activity (see ref. 13). Protease inhibitors were **not** utilized during the preparation of the wheat TFs because previous work (12) indicated that their inclusion decreases the yield of wheat TF activity. This wheat germ will, however, yield an active *in vitro* translation system without inclusion of protease inhibitors (K. Kleene, per. comm.).

An Xho I (−256) to Hind III (+195) fragment of the Ad-2 MLP (36) was sub-cloned into pGem3 to form plasmid pGlemp (35). A Pss I digest of pGlemp produces a 598n run-off RNA. Nci I digests pGlemp in the Ad-2 MLP upstream region (−51) and in the vector to produce a 444n run-off RNA. The CaMV 35S promoter (−421 to +13) in pUC19 was a gift from Dr. T. Ueda. It was re-cloned in the opposite orientation as a Hind III fragment (−421 to +13) in pGem3. Both constructs were used to insure that results were not due to fortuitous initiation at cryptic promoter sites in a vector. Digestion of CaMV/pUC with Ssp I yields a 585n run-off RNA while digestion of CaMV/pGem with Pss I produces a 404n run-off RNA. Neither enzyme cuts in the CaMV sequences.

Transcription reactions were incubated for 60 min (unless indicated otherwise) at 25° or 30°C as indicated, in 25 µl (11,13,35). Each reaction contained 8–10 mM HEPES (pH 7.9), 60 mM KCl, 7.5 mM $MgCl_2$, 2 mM DTT, and 4 mM creatine phosphate. Optimum template concentration was determined for each digest and was always between 20 and 40 µg DNA/ml. HeLa transcription fractions were used at the following volumes: C (5 µl, 4.5 µg), D (4 µl, 1.9 µg), DB (3 µl, ≤1.8 µg), DE (6 µl, 0.9 µg), F (2 µl, 2.5 µg), and FS (7 µl, ≤1.9 µg). Wheat germ fraction KB was used at 4 µl (≤3.6 µg) and KI at 6 µl (≤60 ng). Control reactions (which were included in every assay) omitted TFIIA and instead had an equivalent volume of BSA (0.1 mg/ml) added. Reactions contained 300 µM CTP and UTP, 20 µM GTP, 400 µM ATP, and 5.0 µCi α-^{32}P-GTP. Transcription reactions were processed and analyzed by denaturing (urea) polyacrylamide gel electrophoresis (PAGE) and autoradiography as described (11,13,35). ^{32}P-labelled fragments from an Msp I restriction digest of pBR322 DNA were used as size markers (New England Biolabs). Run-off transcript bands were excised from the gel and quantitated by liquid scintillation counting. Background radiation was determined from a gel slice above or below the band. RNA synthesis was calculated as the percent transcription relative to controls in each experiment. To compare transcription yields of different-sized run-off RNAs we calculated the number of RNA molecules synthesized rather than the total incorporation of radioisotope.

For S1 nuclease analysis, large quantities of unlabelled RNA (reaction volume 125 µl) were synthesized *in vitro*, for each CaMV construct. The run-off RNA was isolated by denaturing PAGE. To allow accurate location of the run-off RNAs, adjacent to each lane of unlabelled RNA was a lane that contained a reaction (25 µl) of synthesized radioactive run-off RNA. RNA was electroeluted (IBI model UEA) from the gel, ethanol precipitated, resuspended in 25 µl TEEN (10 mM Tris-Cl (pH 7.9)-0.1 mM EDTA-0.1 mM EGTA-5 mM NaCl), extracted with phenol:chloroform:iso-amyl alcohol (48:48:1), and with chloroform:iso-amyl alcohol (24:1), and ethanol precipitated. DNA probes were prepared by digesting CaMV/pUC19 with Eco RI (which cuts at +155 from the start site) and CaMV/pGem3 with Rsa I (which cuts at +115). Digests were treated with calf intestinal phosphatase (CIP), incubated at 68°C to inactivate CIP, then extracted and precipitated as above. The CaMV templates were end-labeled by kinase and then digested with Eco RV (which cuts both templates at −90). Appropriate fragments were isolated by agarose gel (1%) electrophoresis, recovered by electroelution, extracted, and ethanol precipitated. Hybridizations were in 10 µl [80% formamide, 3× SSC (1× = 0.15 M NaCl, 0.015 M Na citrate), 7 mM PIPES (pH 6.8), 1 mM EDTA, 1 mM EGTA, and 20 µg tRNA/ml] in sealed 50 µl Drummond microcaps (pre-cleaned by boiling in 1 mM EDTA-1 mM EGTA, washing, and autoclaving in water). After incubation at 95°C for 3 min samples were incubated at 37°C for 26 hr. Samples were then diluted 25-fold with S1 nuclease buffer (10 mM Na succinate, pH 4.5, 300 mM NaCl, 0.5 mM $ZnCl_2$, 25 µg tRNA/ml) and treated with S1 nuclease as described (55): 100 units of S1 nuclease (Miles) was used in each reaction, for 60 min. Samples were then extracted, ethanol precipitated, resuspended in TEEN, and analyzed by denaturing PAGE.

SDS-PAGE was as described (37) using a 4% polyacrylamide (acrylamide: bisacrylamide, 30:0.9) stacking gel and a 10% polyacrylamide (acrylamide:bisacrylamide, 37.5:1) separating gel. Proteins were precipitated with two volumes of acetone (at −20°C or 0°C overnight), centrifuged at 10,000 rpm for 10 min, the pellets dried *in vacuo*, and resuspended in 25 µl of 50 mM Tris-Cl (pH 6.8), 100 mM DTT, 0.2% SDS. Samples and molecular weight markers (Bio Rad) were incubated at 50°C for 1 hr (or overnight at 30°C), dye (75% glycerol, 0.1% bromphenol blue; 2 µl) was then added to each sample, and electrophoresis was at 20 to 25 mA. After electrophoresis the gel was fixed (50% methanol, 5% acetic acid) for 1 hr, then stained with Coomassie Brilliant Blue and for 1 hr at 50°C, and destained in fixer. A BSA lane was included on each gel since BSA had been added to all chromatographic fractions.

Renaturation of TFIIA after SDS-PAGE was as described (65−67). Four ml of fraction KI was acetone precipitated, resuspended, and electrophoresed as above. The 35 kd band was excised from the gel and washed in 0.1 M Tris-Cl (pH 7.9)-0.01 M DTT for 20 min (66). The solution was removed and the slice was placed in 1 ml of 50 mM Tris-Cl (pH 7.9)-0.1 mM EDTA-0.1% SDS-5 mM DTT-200 mM NaCl (65). The gel was crushed with a motor driven teflon pestle (at 4°C). After incubation at 21°C for 4 hr with shaking the acrylamide was removed by centrifugation (65). To the supernatant 5 µg of BSA and five volumes of acetone were added and precipitation permitted for 18 hr at −20°C. Precipitated protein was collected by centrifugation (14500×g for 30 min). The pellet was resuspended in 100 µl of buffer B which contained 5 mM DTT, 100 µg BSA/ml, and 6 M guanidine-HCl. SDS and guanidine-HCl were then removed by passing the solution through a 400 µl column (in a blue micropipet tip) of Bio-Rad P-10 (65). Fractions of 300 µl were collected into tubes containing 50 µg BSA. Refolding of the protein was allowed to occur for 1 hr at 21°C and then at 4°C for 3 hr. Fractions were assayed for activity as described above.

RESULTS

Purification of wheat germ TFIIA

Partially purified wheat TFIIA (fractions F, K, KD, and KB) has previously been shown to substitute for human TFIIA in an *in vitro* transcription system that contains HeLa fractions C and DE (TFIID) (ref. 13 and Fig. 2). Omission of HeLa TFIIA decreases transcription to background levels (lane 2). If HeLa TFIID or fraction C are omitted when TFIIA is present there are no detectable run-off RNAs (not shown). To further purify wheat TFIIA, fraction KB was subjected to gel filtration (Fig. 3). Transcription activity of TFIIA activity was predominantly observed in two peaks; the first included fractions 25−33 and coincided with the excluded protein peak. TFIIA activity in this region of the chromatogram may be due to association of TFIIA with other TFs (12). A second peak of protein was observed around fraction 50; this corresponded to the BSA added during previous purification steps to stabilize TFIIA. A second peak of transcription activity was seen in fractions 53−57, which

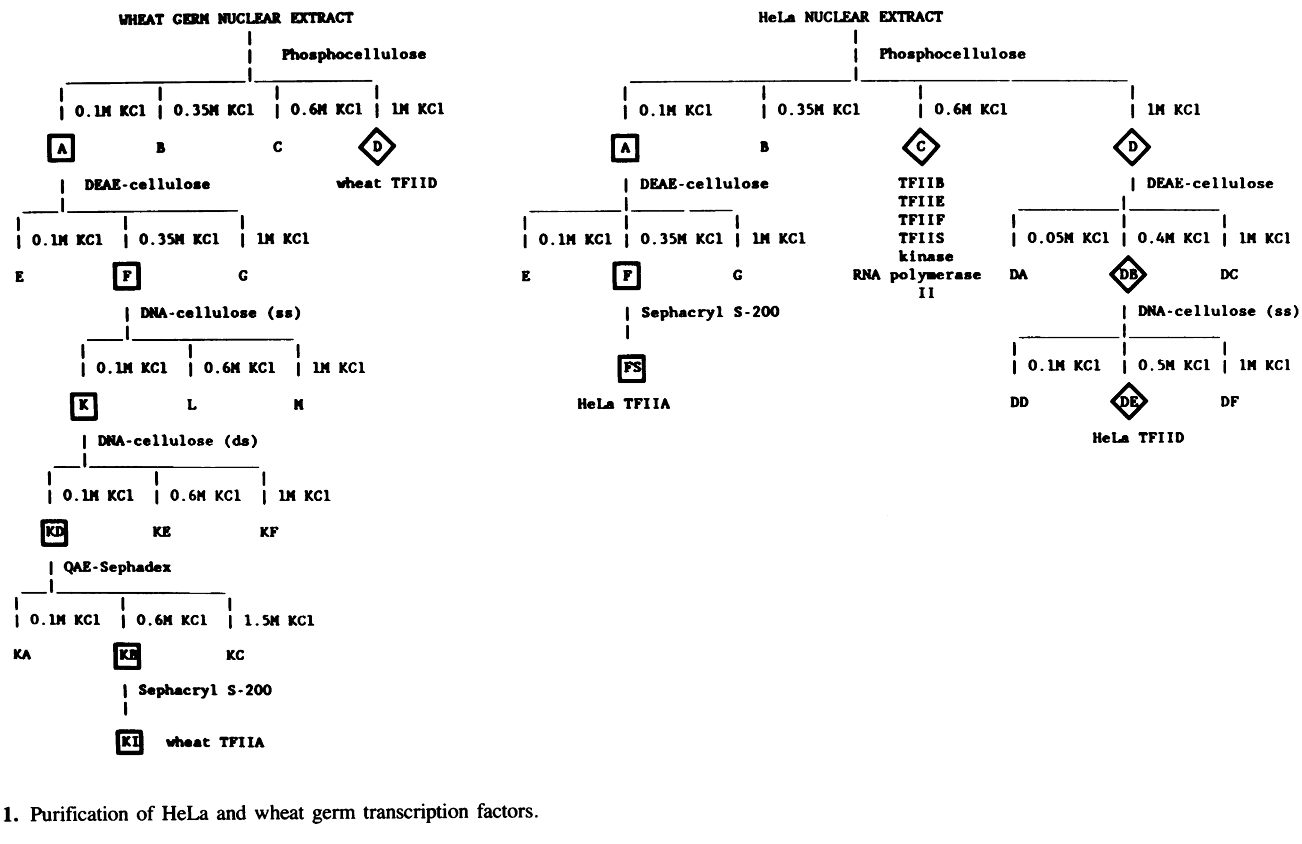

Fig. 1. Purification of HeLa and wheat germ transcription factors.

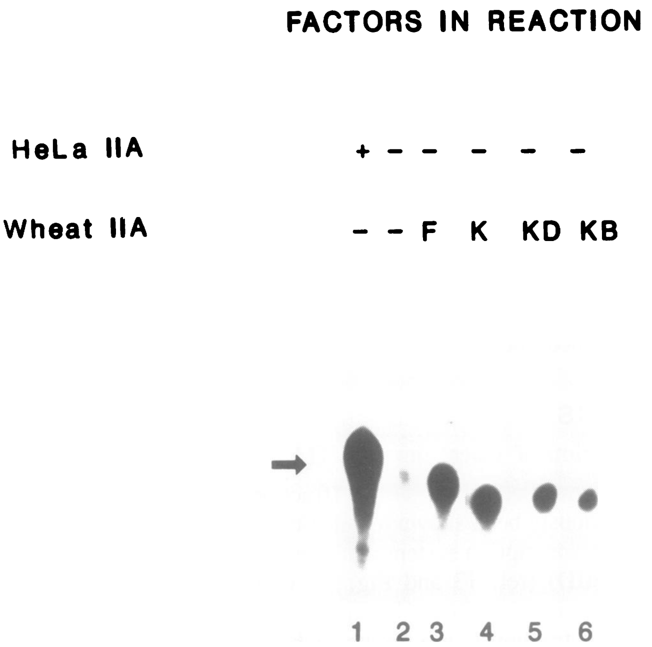

Fig. 2. Wheat TFIIA substitution in the HeLa system. Details are described in Methods; the template used was Ad-2 MLP digested with Pss I, and the run-off RNA (arrow) was 598n. The amounts of synthesized RNA (always referring to run-off RNA) by each reaction were (in f moles): HeLa, 336; wheat F/HeLa, 156 (46%); wheat K/HeLa, 140 (42%); wheat KD/HeLa, 64 (19%); wheat KB/HeLa, 56 (17%). Lane 2 contained, in lieu of HeLa TFIIA, 7 μl of BSA (0.1 mg/ml).

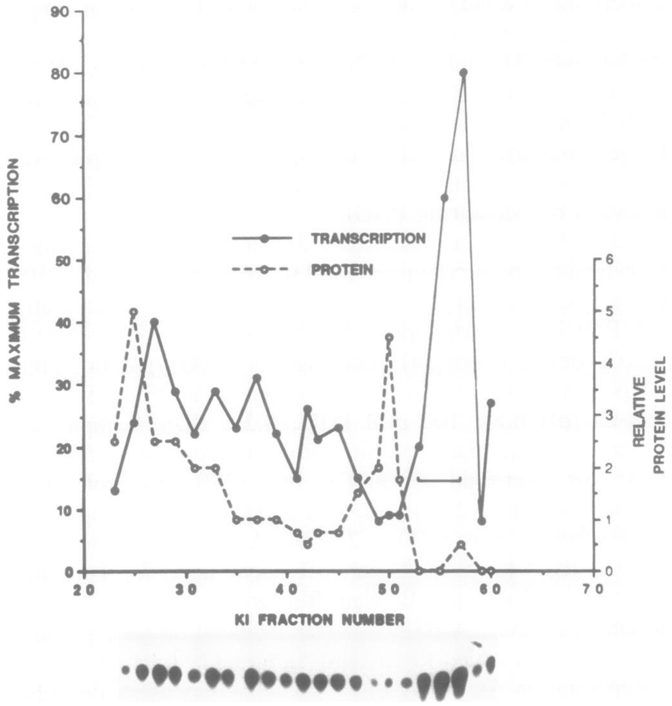

Fig. 3. Gel filtration chromatogram of wheat fraction KB on Sephacryl S-200. The auto-radiogram of the gel of the transcription activity assay of odd numbered fractions (and fraction 50) is depicted below the chromatogram. Only the region of the gel that contained the run-off RNA is presented. The template was Ad-2 MLP digested with Pss I. Fractions 53–57 were pooled and are referred to as fraction KI. The HeLa system synthesized 584 f moles RNA (not shown) and fraction 57 synthesized 236 f moles (40%). 100% transcription activity (fraction KB) was the standard to which the fractions were compared. The protein concentration is relative to the BSA peak.

contained very little protein. These fractions were combined (fraction KI) and represent purified TFIIA (Fig. 3). The purification of wheat germ TFIIA through fraction KI resulted in at least a 5000-fold purification, with 12% recovery of the transcription activity of fraction F (Table I).

To estimate the size of wheat TFIIA, fraction KI was analyzed by SDS-PAGE (Fig. 4A, lane 3). Most of the bands are due to BSA (lane 2), including high and low mw polypeptides attributable to aggregates and degradation products of BSA, respectively. To reduce the BSA contribution TFIIA was prepared as above but following the gel filtration step BSA was **not** added (lane 4). Although residual BSA still remains, both TFIIA preparations show only one common polypeptide at ~35 kd. The doublet band at ~31 kd in lane 3 is not seen in lane 4 or in several other preparations (also cf. ref. 38). Polypeptides present in the peak fractions (49–51) exhibited a pattern similar to fraction KB after SDS-PAGE (data not shown).

The band at ~35 kd was eluted from the gel, renatured, and assayed for function (Fig. 4B). The HeLa system depleted of TFIIA will support transcription from the Ad-2 MLP when the refolded wheat TFIIA is added (lanes 6–9). Fractions eluting earlier (lanes 4,5) or later (lanes 10,11) do not reconstitute the transcription system (lane 3). We estimate that the recovery of activity is about 4%, consistent with results obtained with other transcription factors (65,66). Similar results were observed for transcription using the cauliflower mosaic virus 35S promoter (Fig. 9B), where the estimated recovery of activity was 6%.

Enzymology of wheat TFIIA

We next examined optimal conditions for wheat TFIIA in the heterologous *in vitro* transcription system. HeLa fractions C and DE were used with either HeLa TFIIA (fraction FS), or wheat fraction KB or KI.

Maximal levels of transcription for the wheat/HeLa system are at 25°C, while maximal transcription for the HeLa system occurs at 30°C (Fig. 5A). In subsequent experiments the incubation temperature was maintained at 25°C to optimize transcription for wheat TFIIA.

The optimal KCl concentration is similar for both systems. For the wheat/HeLa system maximal transcription is observed at 60 mM (fraction KB) to 75 mM (fraction KI) KCl, while maximal levels for the HeLa system are at 60 mM KCl (Fig. 5B).

Template concentration for optimal transcription is also similar for both systems. Maximal transcription for the wheat/HeLa system is observed between 20 μg/ml (fraction KI) to 40 μg/ml (fraction KB), and at 40 μg/ml with the HeLa system (Fig. 5C).

Optimal $MgCl_2$ concentration for maximal transcription activity for the wheat/HeLa system is at 7.5 mM (KB) to 10 mM (KI) $MgCl_2$ while that for the HeLa system is 7.5 mM $MgCl_2$ (Fig. 5D).

Our standard template (pGlemp digested with Pss I) includes upstream sequences of Ad-2 MLP to −256, and exhibits a lag period of ~20 min with both wheat/HeLa and HeLa systems (Fig. 6A and ref. 25), during which assembly of the transcription complex occurs. Digestion of pGlemp with Nci I maintains the TATA box (−30) but removes sequences upstream of −51, resulting in the minimal promoter. With this second template the lag period is decreased in both systems (Fig. 6B). The kinetics of RNA accumulation, however, differ between the two systems.

TABLE I

Fraction	Protein (mg/ml)	Total volume	Total protein	%[6] protein	Assay[7] volume	RNA per rx. (fmoles)	Total[8] units	% Activity
nuclear	22.8	70 ml	1592 mg	100	---	---	---	---
A	6.4	125 ml	800 mg	50	---	---	---	---
F[1]	3.2	50 ml	210 mg	13	2 ul	38.7	1273	100
K[2]	2.8	40 ml	184 mg	11	2 ul	35.4	1164	91
KD[3]	0.6	36 ml	39 mg	2	2 ul	16.6	546	43
KB[4]	0.9	13 ml	24 mg	1.5	4 ul	28.1	187	15
KI[5]	≤0.01	15 ml	≤0.33 mg	≤0.02	6 ul	26.9	150	12

[1] 96 ml of fraction A loaded on DE52; 76% of total
[2] 40 ml of fraction F loaded on DNA cellulose; 80% of total
[3] 36 ml of fraction K loaded on DNA cellulose; 90% of total
[4] 32 ml of fraction KD loaded on QAE; 89% of total
[5] 12 ml of fraction KB loaded on Sephacryl; 92% of total
[6] amount of protein in fraction compared to amount in nuclear extract
[7] volume of fraction added to in vitro transcription reaction
[8] 1 unit = 1 pmole of RNA synthesized in 60 min at 25°C

The early kinetics are slower for the wheat/HeLa system compared to the HeLa system, but later (between 60–120 min with the long upstream template, Fig. 6A, or 30–60 min for the short upstream template, Fig. 6B) the rate of RNA accumulation in the wheat/HeLa system is greater.

HeLa TFIIA first associates with the Ad-2 MLP followed by or coincidental with TFIID, and this pre-formed IIA/IID/DNA complex initiates transcription rapidly when the remaining components are added (5,8,25). Pre-incubation experiments indicate that wheat TFIIA also participates in the early stages of transcription complex assembly (Fig. 7). TFs IIA and IID were pre-incubated with the Ad-2 MLP for various times, then the remaining components were added and transcription permitted for 15 min (to allow synthesis only from pre-formed complexes). We observe a similar rate of RNA accumulation for both systems suggesting that wheat TFIIA participates in the initial steps of transcription complex assembly and that both systems have similar elongation rates.

The temperature stability of TFIIA was also examined (Fig. 8). Both HeLa and wheat TFIIA are unaffected by a 15 min pre-incubation at 0°C, and transcription activity remains at >80% of control activity following pre-incubation at 23°C and 25°C. Pre-incubation of both TFIIAs at 30°C or higher results in significantly decreased transcription; wheat TFIIA pre-incubated at 80°C decreases transcription activity to 60% of maximum, while HeLa TFIIA decreases to 45% of maximum. Similar results were obtained with the renatured TFIIA (data not shown).

A plant viral promoter works in a human system

The purified wheat TFIIA was used in a heterologous system with the cauliflower mosaic virus (CaMV) 35S promoter. We selected the CaMV 35S promoter because it is often used to analyze plant gene expression (39–42) and is transcribed by the HeLa system (45,68). Two different constructs containing the CaMV promoter were prepared (Methods and Fig. 10), one in pGem3, digested with Pss I, the other in pUC19, digested with Ssp I. With both constructs the expected size run-off RNAs are observed in both transcription systems (Fig. 10) and are α-amanitin (1 μg/ml) sensitive (not shown). There is only a short lag period, with transcripts observed between 5 and 10 min, and RNA synthesis continues for at least 120 min (Fig. 9A). Wheat TFIIA activity is still less than HeLa TFIIA, but interestingly the kinetic differences observed between the wheat/HeLa and HeLa systems with the Ad-2 MLP are not seen with the CaMV promoter. Renatured wheat TFIIA also functions with the CaMV 35S promoter (Fig. 9B).

To demonstrate that RNA synthesis was directed by the CaMV promoter S1 nuclease analysis was conducted. From previous detailed studies mapping the transcription start site (39,43–46), the expected size of the protected probes after S1 nuclease digestion was 115n for the CaMV/pGem3 probe and 155n for the CaMV/pUC19 probe. Gel purified RNA from *in vitro* transcription of the two CaMV constructs was hybridized to the appropriate probe and S1 nuclease digested. The protected probe fragment was of the expected size, indicating that initiation is directed by the CaMV promoter at or near the start site (Fig 10). The CaMV/pGem3 probe does not protect RNA synthesized from the CaMV/pUC19 template, and vice-versa (data not shown).

DISCUSSION

The development of a plant *in vitro* transcription system remains elusive. RNA polymerase II **run-on** transcription (but not initiation) by turnip leaf nuclei containing CaMV mini-

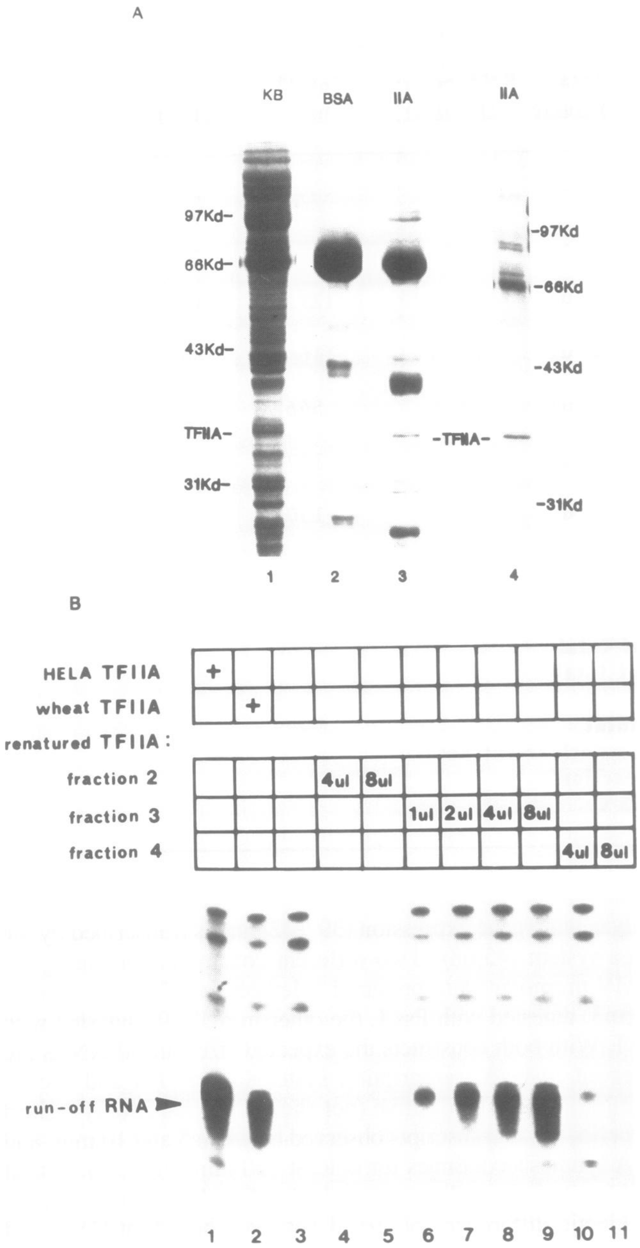

Fig. 4. (A) SDS-PAGE analysis of wheat germ fractions KB and KI. Lane 1 is fraction KB. Lane 2 is BSA. Lane 3 is fraction KI; the fractions comprising KI contained BSA; 250 μl of fraction KI was used for this analysis. Lane 4 is fraction KI to which BSA was *not* added after the last step; 400 μl of fraction KI was used for this analysis (the transcriptional activity in this preparation was lost very rapidly). Although BSA elutes prior to TFIIA (Fig. 3) contaminating BSA is still present in the TFIIA preparation (lane 4). (B) Renatured TFIIA was assayed with the Ad2-MLP. The eluted fractions from the Bio-Rad P-10 column were individually tested for transcription activity; fraction 3 contains >95% of this activity. The protein concentration of the renatured TFIIA is mainly BSA; we estimate that the TFIIA is present at less than 3 μg in fraction 3; 1 μl would be no more than 10 ng of TFIIA. The amounts of RNA synthesized were (in f moles): Lane 1 (HeLa), 75; lane 2 (wheat), 45 (60%); lane 8 (renatured TFIIA), 43 (57%); lane 9 (renatured TFIIA), 55 (73%).

chromosomes has been reported (47). A soluble, reconstituted wheat RNA polymerase II system was reported to utilize an octopine promoter of a Ti plasmid (48) and a fractionated, reconstituted tobacco RNA polymerase II system was reported to utilize the CaMV 19S promoter (68). We observed that although wheat nuclear extracts initiate transcription by RNA polymerase II on the Ad-2 MLP, they do not elongate RNA (12). Similarly, the tobacco system did not elongate RNAs initiated at the CaMV 35S promoter (68). It appears that there are transcription inhibitors present which can only be removed by further purification of each transcription factor (68). We partially purified two wheat proteins from our nuclear extract, and showed that they substitute for HeLa TFIIA and TFIID, respectively (13). Wheat TFIIA also increased the processitivity of wheat RNA polymerase II (26).

Wheat germ TFIIA has now been purified and characterized. Overall, it is very similar to HeLa TFIIA in many enzymic properties. (Data concerning DNA:TFIIA interactions will be published separately, ref. 69). We can thus extend the comparison of functional homology between wheat and HeLa TFIIA:

1) HeLa and wheat TFIIA both chromatograph similarly on phosphocellulose, DEAE-cellulose, and Sephacryl (Figs. 1 and 3; refs. 13,35). Wheat germ TFIIA was purified at least 5000-fold, which is a minimum estimate because most of the protein in the Sephacryl column-purified TFIIA is BSA. Activity in the nuclear extract and fraction A cannot be determined because of transcription inhibitors (12) but there was recovery of 12% of fraction F activity. TFIIA activity was lost very rapidly without inclusion of the BSA (see Methods).

By SDS-PAGE analysis wheat TFIIA has an M_r of ~35 kd. Partially purified HeLa TFIIA was reported to have an M_r of ~84 kd as determined by gel filtration (8) but we have observed that HeLa TFIIA also elutes after BSA (66 kd) (35; Kundzicz and Ackerman, unpublished) and may be smaller than wheat TFIIA (69). (A size disparity between human and yeast TFIID has also been observed (eg. 49).) Calf thymus TFIIA (23) may be two polypeptides of combined M_r ~32 kd, a size very similar to that of wheat TFIIA.

The 35 kd polypeptide can be eluted from the denaturing gel, refolded, and transcription activity restored (figs. 4B and 9B).

2) Wheat TFIIA substitutes for HeLa TFIIA in a human transcription system containing the Ad-2 MLP. Transcription efficiency with the wheat TFIIA is, however, only 30–60% that of the HeLa system. This lower efficiency is probably not due to inactivation during purification since less purified fractions (F, K, KD, KB) (Fig. 2; ref. 13) do not substitute with greater efficiency. Possibly the wide divergence between the species may have resulted in proteins that are inherently different in activity.

3) Wheat TFIIA functions in a HeLa *in vitro* transcription system with reaction parameters (KCl, $MgCl_2$, and DNA concentrations) similar to HeLa TFIIA (Fig. 5). The optimal incubation temperature with the wheat TFIIA is slightly lower, 25°C, than with HeLa TFIIA (30°C) (Fig. 5). This may relate to the optimal growth of wheat at lower temperatures. Both TFIIA proteins exhibited similar sensitivities to heat inactivation (Fig. 8).

4) Wheat and HeLa TFIIA interact similarly with HeLa TFIID and/or DNA at early stages of initiation, specifically pre-initiation, and have similar elongation rates (Fig. 7). Different transcription kinetics with the Ad-2 MLP are observed with the wheat/HeLa and HeLa systems (Fig. 6). The HeLa system exhibits faster initial rates of RNA accumulation while at later times the rate of RNA accumulation is faster with the wheat/HeLa system. Since the elongation rates of the two systems are similar (Fig. 7) we believe that the difference may be due to slower kinetics of initiation in the wheat/HeLa system. Possibly the wheat TFIIA is not completely compatible with the HeLa system. The later 'burst' of RNA accumulation would result once the slow initiation

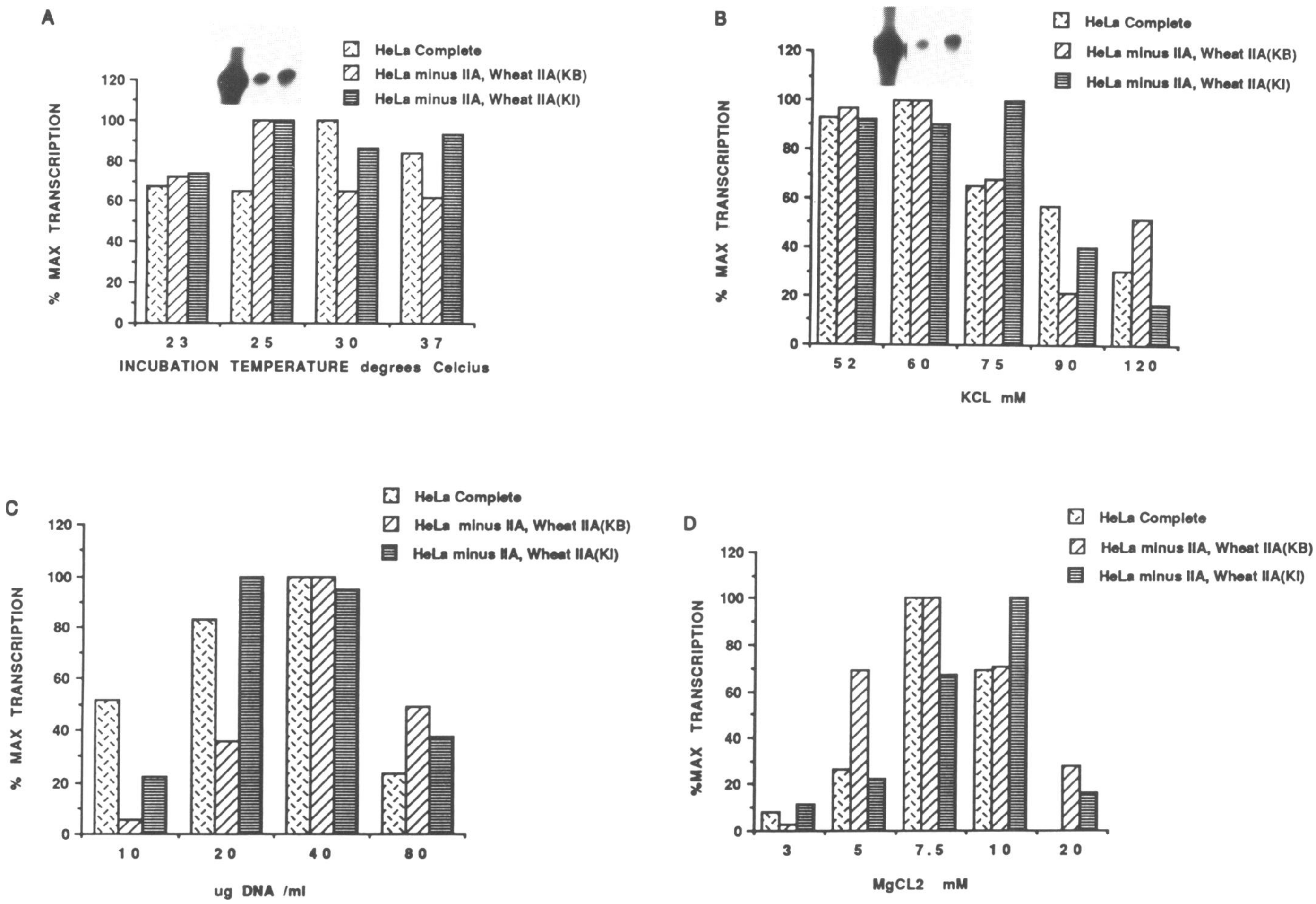

Fig. 5. Kinetic properties of the wheat/HeLa transcription system. For each of the panels in this figure transcription using wheat TFIIA decreased the run-off RNA yield to 35–60% of the HeLa level as monitored during at least three separate assays for each panel. For example, in (A) at 25°C the HeLa system synthesized 136 f moles of RNA and the wheat KI/HeLa system synthesized 72 f moles (53%). The template used was the Ad-2 MLP digested with Pss I. (A) Transcription incubation at different temperatures. A representative autoradiogram for the incubations at 25°C is included as an insert. Reactions containing the wheat/HeLa system had their optimal transcription level (25°C) set to 100% and the transcription yield of run-off RNA at the other temperatures was compared to this value. For the HeLa system 100% was at 30°C. (B) KCl concentration optima. Details of the analysis are the same as described for (A). The insert autoradiogram is the transcription with 60 mM KCl. (C) DNA concentration optima. (D) $MgCl_2$ concentration optima.

events had occurred.

The kinetic experiments also showed similarities in lag times for both systems (Fig. 6). With the complete Ad-2 MLP upstream region both the wheat/HeLa and HeLa systems exhibit a lag period of ~20 min, and removal of upstream sequences eliminates the lag. This is probably due to a protein (MLTF), which binds to upstream sequences of the Ad2-MLP at −66/−51 (50–53) and affects transcription rates (21,54,56). With the minimal promoter (lacking sequences upstream of −50) unfractionated extracts (containing MLTF protein) exhibited decreased transcription (50,52,53,57) and increased lag times (54). But the minimal promoter in a reconstituted system **lacking** MLTF protein (but still including TFs IIA, IIB, IID, IIE, IIF, and RNA polymerase II) exhibited normal transcription (19,52) and short lag times (54). In our system MLTF protein is also not present and we observe rapid transcription using a minimal promoter (Fig. 6B). We also observe that with a longer promoter there is a long lag (Fig. 6A). Thus the MLTF binding region, when not occupied by MLTF protein, causes a long lag and decreased transcription.

5) We observed that a plant viral promoter, the CaMV 35S promoter, is accurately and efficiently utilized by both the HeLa and wheat/HeLa *in vitro* transcription systems (Fig. 9). S1 nuclease analysis of the *in vitro* transcripts indicates transcription originates from the CaMV promoter (Fig. 10). The CaMV-containing template is transcribed without the long lag we observe with the Ad-2 MLP containing upstream sequences. The CaMV upstream sequences (−421 to +1), which contain all the necessary sequences for *in vivo* gene expression (39,40), have been delineated into several domains, each with a specific *in vivo* function (41,42,58). There is a TATATAA sequence at −28, a CCACT sequence at −54, and a CAAT sequence at −61; upstream deletions to −90 exhibit decreased transcription efficiency *in vivo* (41,42) but **not** *in vitro* (S. Sif and Ackerman, unpublished). Experiments are now in progress to determine if the *in vivo* domains are similar in the *in vitro* system.

The kinetic differences between the wheat/HeLa and HeLa systems with the Ad-2 MLP are not observed with the CaMV promoter. This may be related to the plant origin of the CaMV, or to some other difference between the promoters. Although

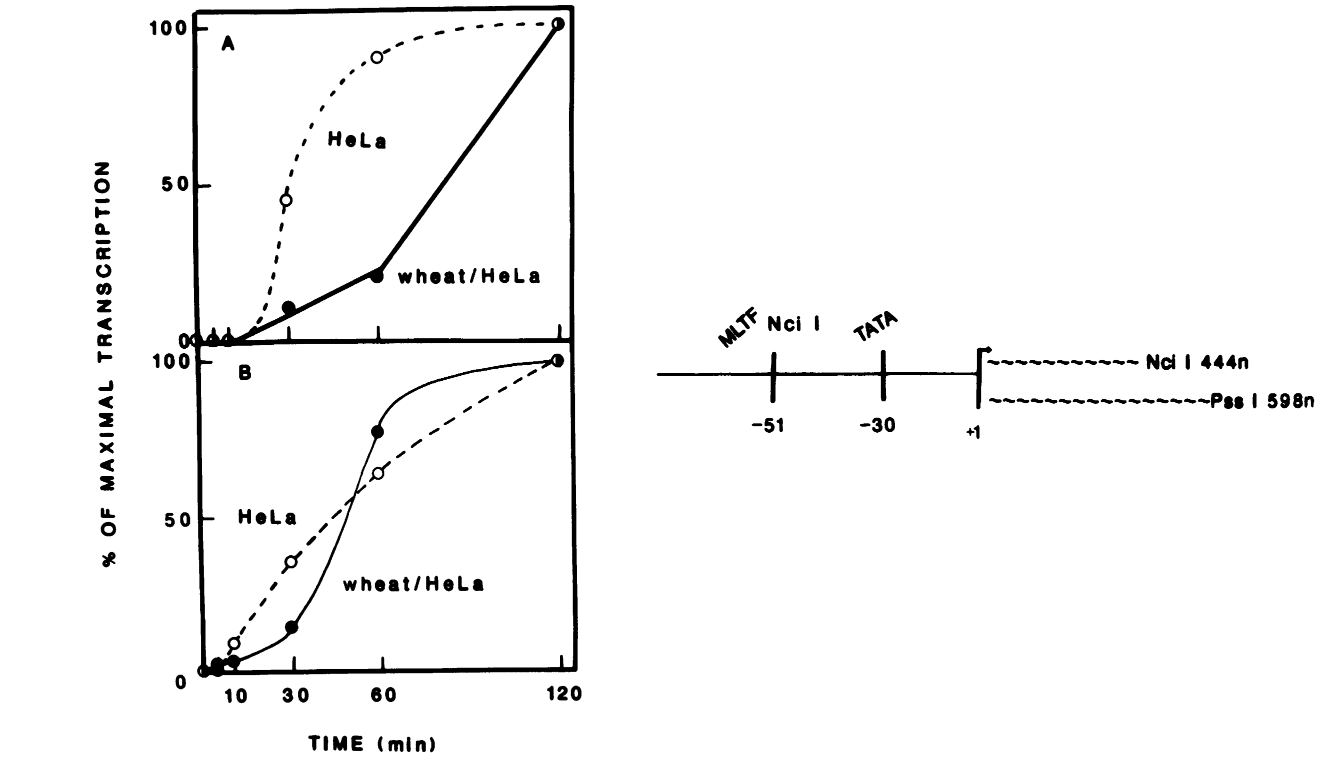

Fig. 6. Kinetics of transcription of the Ad-2 MLP by the wheat/HeLa system. When the template contains (A) or lacks (B) sequences upstream of −51 (the Ad-2 MLP was digested with Pss I or with Nci I) two different run-off RNAs resulted. This is depicted on the right. (A) The HeLa system synthesized 780 f moles RNA (5.2 f moles of initiations) while the wheat/HeLa system synthesized 408 f moles (2.7 f moles of initiations; 52%). (B) The HeLa system synthesized 1808 f moles RNA (16.3 f moles of initiation) while the wheat/HeLa system synthesized 900 f moles (8.1 f moles of initiations; 50%). The wheat/HeLa system contained TFIIA from fraction KI. The run-off RNA yield from the 120 min reaction for each set was taken as 100% and the yields at the other time points compared to that amount. Transcription from the Nci I-digested Ad-2 MLP (calculated as n moles of molecules synthesized) was always at least 3-fold greater than that from the Pss I-digested template.

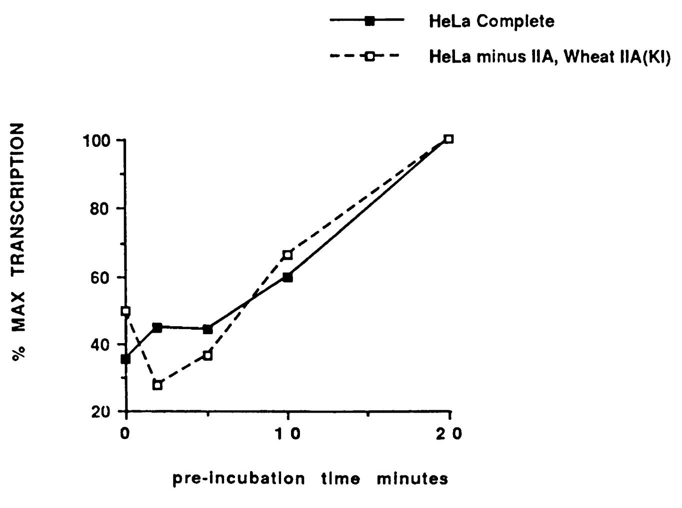

Fig. 7. Kinetics of transcription following pre-incubation of TFIIA, TFIID, and Ad-2 MLP. Wheat fraction KI or HeLa TFIIA were pre-incubated with HeLa TFIID and Ad-2 MLP. Following pre-incubation at 25°C (in a 14 μl volume that maintained normal transcription assay conditions) HeLa fraction C and rNTPs were added, the volume increased to 25 μl (again maintaining normal transcription assay conditions), and incubation continued at 25°C for 15 min. The yield of run-off RNA from the 20 min pre-incubation for each set was taken as 100% and the other time points compared to that value.

Fig. 8. Heat inactivation of TFIIA. Wheat TFIIA (fraction KI) and HeLa TFIIA were pre-incubated at the indicated temperatures (0°C, 23°C, 25°C, 30°C, 42°C, 50°C, and 80°C) for 15 min and then added to a transcription reaction that contained all remaining components. Pre-incubation of TFIIA at 0°C resulted in the highest yield of RNA for each set and was used to normalize the other values. The HeLa system synthesized 656 f moles of run-off RNA and the wheat/HeLa system synthesized 352 f moles.

the HeLa system is more efficient with the CaMV promoter than the wheat/HeLa system, the kinetics indicate that the two systems initiate, and elongate, with equivalent rates (Fig. 9).

Plant promoters have been shown to function in other animal systems. A maize zein promoter is utilized in an *in vitro* HeLa system (59,60) or *in vivo* in yeast cells (61,62), an alcohol dehydrogenase 1 promoter functions in mammalian cells (63), and the CaMV promoter functions in HeLa *in vitro* systems (45,64,68). Animal promoters, however, do not function in plant cells (reviewed in ref. 64).

These similarities extend our previous work (12,13) and

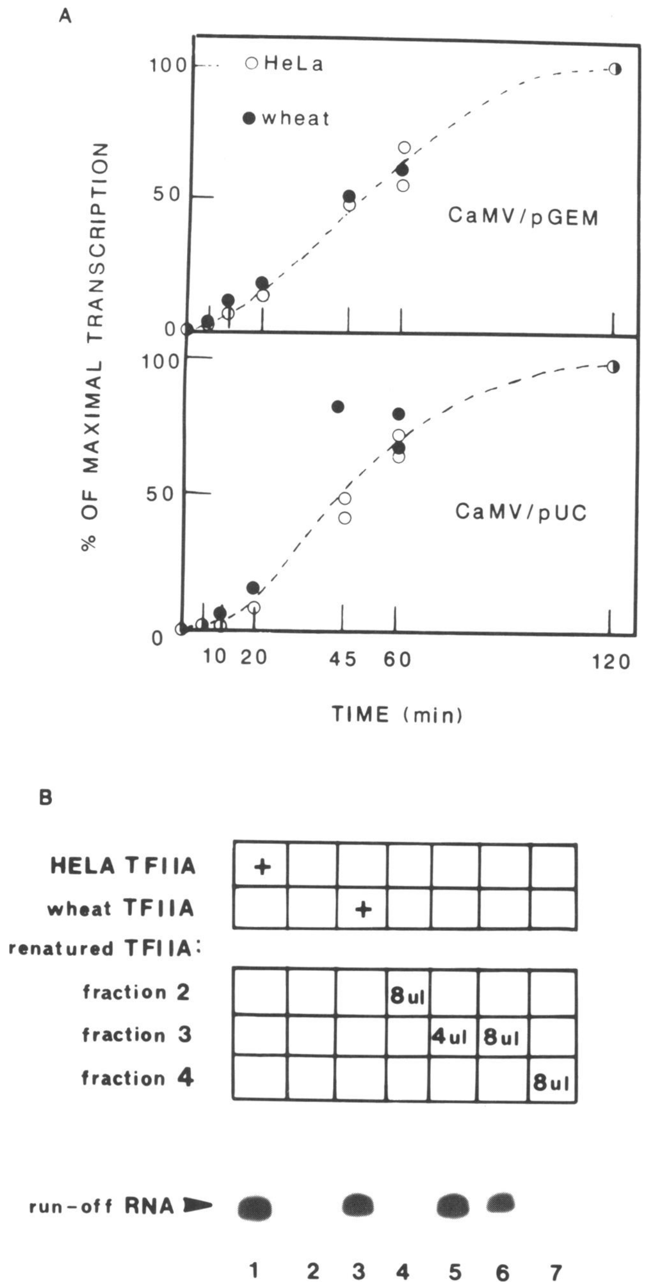

Fig. 9. (A) Transcription driven by the CaMV 35S promoter in the wheat/HeLa and HeLa systems. The CaMV 35S construct in pUC19 or pGem3 was digested with Ssp I or Pss I, respectively. Standard transcription reactions contained the pre-determined optimal concentration of each template. The yield of run-off RNAs produced by the 120 min incubation were taken as 100% and the yields at earlier time points for each set were compared to that value. The CaMV 35S promoter-driven transcription varied between 35 and 125% of the (Nci I digested) Ad-2 MLP-driven transcription in different repeats of the assays (using either system), but the kinetics were identical. For example, in one experiment using the HeLa system the CaMV/pUC template (167 f moles RNA; 1.1 f moles of initiations) was 33% as efficient as the Ad-2 MLP while the CaMV/pGem template (134 f moles RNA; 1.3 f moles of initiations) was 39% as efficient; the Ad2-MLP reaction synthesized 372 f moles of RNA (3.3 f moles of initiations). (B) Renatured TFIIA was assayed with the CaMV 35S promoter construct digested with Pss I. The eluted fractions from the Bio-Rad P-10 column were individually tested for transcription activity; fraction 3 contains >95% of this activity. The amounts of synthesized RNA (in f moles) were: Lane 1 (HeLa), 50; lane 3 (wheat), 45 (90%); lane 5 (renatured TFIIA), 56 (112%). In comparison to the Ad-2 MLP (Fig. 4B) the CaMV 35S promoter was (after correcting for the size difference between the run-off RNAs) 72% as efficient with the HeLa system, 109% as efficient as the Ad-2 MLP with the wheat/HeLa system, and 112% as efficient as the Ad-2 MLP with the renatured wheat TFIIA/HeLa system.

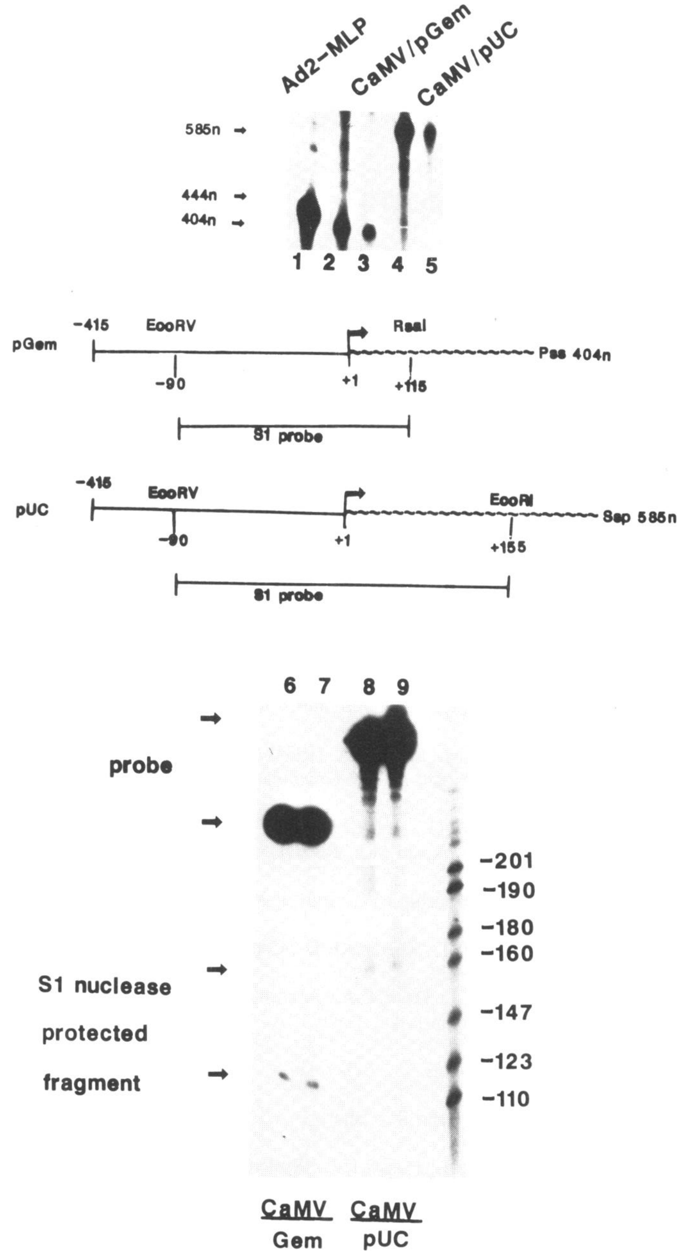

Fig. 10. S1 nuclease analysis of the CaMV 35S-driven RNA products. The top part of the figure depicts the transcription products from each template. Lane 1 is RNA synthesized (544 f moles; 4.9 f moles of initiations) by the HeLa system using the Nci I digested Ad-2 MLP. Lanes 2 and 3 are the transcripts from the CaMV/pGem construct: lane 2 is RNA synthesized (196 f moles; 1.9 f moles of initiations) by the HeLa system and lane 3 contained RNA synthesized (136 f moles, 1.3 f moles of initiations) by the wheat/HeLa system. Lanes 4 and 5 are the transcripts from the CaMV/pUC construct: lane 4 is RNA synthesized (248 f moles; 1.7 f moles of initiations) by the HeLa system and lane 5 is RNA synthesized by the wheat/HeLa system. For S1 analysis RNA was unlabelled (see Methods). The construct and the run-off RNA size are shown in the middle part of the figure, with the S1 probe and the anticipated size of the protected probe after S1 nuclease digestion. The lower part of the figure shows the S1 nuclease-protected fragments on denaturing 6% PAGE. Lanes 6 and 7 are the RNA transcripts from the CaMV/pGem construct, and are the same RNAs as in lanes 2 and 3, respectively, above. Lanes 8 and 9 are the RNA transcripts from the CaMV/pUC construct, and are the same RNAs as in lanes 4 and 5, respectively, above.

support the concept that regulation of gene expression at the level of transcription initiation may be very ancient. Since plants and animals diverged between 1.5 to 1.1 billion years ago (34), it seems possible that the most basic processes of transcription which involve TFs have been conserved for over one billion years.

ACKNOWLEDGEMENTS

We thank C. King for assistance with photography, N. Scholz for assistance with artwork, Dr. M. Sugumaran for comments about the kinetics, and Drs. P. Higgins and K. Kleene for critical reading of the manuscript. This work was supported by NSF grant (to SA) DMB 8608207, and the following grants (to SA) from the University of Massachusetts: Faculty Development Grants (1-04305, 1-04055, 1-06568), Educational Needs Grants (4-42001: 770471, 770481, 770471), and NIH-sponsored Biomedical Research Support Grants (2SO7RR07199: 632407-07, 632413-08, 632416-08, 632430-09, 632436-10).

REFERENCES

1. Glover,C.V.C.III. (1989) Plant Molec. Biology Reporter, **7(3)**,183–208.
2. Mitchell,P.J. and Tjian,R. (1989) Science, **245**,371–378.
3. Saltzman,A.G. and Weinmann,R. (1989) The FASEB J., **3**,1723–1733.
4. Davison,B.L., Egly,J.M., Mulvihill,E.R., Chambon,P. (1983) Nature, **301**,680–686.
5. Fire,A., Samuels,M., Sharp,P.A. (1984) J. Biol. Chem., **259**,2509–2516.
6. Burton,Z.F., Ortolan,L.G., Greenblatt,J. (1986) The EMBO J., **5**,2923–2930.
7. Khalili,K., Khoury,G., Brady,J. (1986) Virol., **60**,935–942.
8. Reinberg,D., Horikoshi,M., Roeder,R.G. (1987) J. Biol. Chem., **262**,3322–3330.
9. Zheng,X.-M., Moncollin,V., Egly,J.-M., Chambon,P. (1987) Cell, **50**,301–308.
10. Luse,D.S., Kochel,T., Kuempel,E.D., Coppola,J.A., Cai,H. (1987) J. Biol. Chem., **262**,289–297.
11. Logan,K., Zhang,J., Davis,E.A., Ackerman,S. (1989) DNA, **8**,595–604.
12. Ackerman,S., Flynn,P.A., Davis,E.A. (1987) Plant Molec. Biology, **9**,147–158.
13. Flynn,P.A., Davis,E.A., Ackerman,S. (1987) Plant Molec. Biology, **9**,159–169.
14. Buratowski,S., Hahn,S., Sharp,P.A., Guarante,L. (1988) Nature, **334**,37–42.
15. Cavallini,B., Huet,J., Plassat,J.-L., Sentenac,A., Egly,J.-M., Chambon,P. (1988) Nature, **334**,77–80.
16. Lue,N.F., Flanigan,P.M., Sugimoto,K., Kornberg, R.D. (1989) Science, **246**,661–664.
17. Job,D., Soulie,J.-M., Job,C. (1989) Plant Physiol. Biochem., **27(1)**,135–149.
18. Reinberg,D. and Roeder,R.G. (1987) J. Biol. Chem., **262**,3310–3321.
19. Samuels,M., Fire,A., Sharp,P.A. (1982) J. Biol. Chem., **257**,14419–14427.
20. Sopta,M., Carthew,R.W., Greenblatt,J. (1985) J. Biol. Chem., **260**,10353–10360.
21. Reinberg,D., Flores,O., Buckbinder,L. (1987) In Inouye,M. and Dudock,B.S. (eds.),Molecular Biology of RNA: New Perspectives. Academic Press, Inc., New York. pp. 423–440.
22. Flores,O., Maldonado,E., Burton,Z., Greenblatt,J., Reinberg,D. (1988) J. Biol. Chem. **263**,10812–10816.
23. Samuels,M. and Sharp,P.A. (1986) J. Biol. Chem., **261**,2003–2013.
24. Buratowski,S., Hahn,S., Guarante,L., Sharp,P.A. (1989) Cell, **56**,549–561.
25. Zhang,J., Logan,K., Yu,X.-B., Posner,M., Davis,E.A., Ackerman,S. (1990) Life Sci., **46**,729–735.
26. de Mercoyrol,L., Job,C., Ackerman,S., Job,D. (1989) Plant Sci., **64**,31–38.
27. Hahn,S., Buratowski,S., Sharp,P.A., Guarante,L. (1989) The EMBO J., **8**,3379–3382.
28. Durand,R., Job,C., Teissere,M., Job,D. (1982) FEBS Lett., **150**,477–481.
29. Hai,T., Horikoshi,M., Roeder,R.G., Green,M.R. (1988) Cell, **54**,1043–1051.
30. Green,P.J., Yong,M.-H., Cuozzo,M., Kano-Murakami,Y., Silverstein,P., Chua,N.-H. (1988) The EMBO J., **7**,4035–4044.
31. Katagiri,F., Lam,E., Chua,N.-H. (1989) Nature, **340**,727–730.
32. Datta,N. and Cashmore,A.R. (1989) The Plant Cell, **1**,1069–1077.
33. Tabata,T., Takase,H., Takayama,S., Mikami,K., Nakatsuka,A., Kawata,T., Nakayama,T., Iwabuchi,M. (1989) Science, **245**,965–967.
34. Dayhoff,M.O. (1969) Scient. Am., **221**,86–95.
35. Kundzicz,H., Davis,E.A., Ackerman,S. (1989) Bioch. Biophys. Res. Comm., **162**,1133–1139.
36. Weil,P.A., Luse,D.S., Segall,J., Roeder,R.G. (1979) Cell, **18**,469–484.
37. Maniatis,T., Fritsch,E.F., Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
38. Schmidt,M.C., Kao,C.C., Pei,R., Berk,A.J. (1989) Proc. Natl. Acad. Sci. USA, **86**,7785–7789.
39. Odell,J.T., Nagy,F., Chua,N.-H. (1985) Nature, **313**,810–812.
40. Fromm,M.E., Taylor,L.P., Walbot,V. (1986) Nature, **319**,791–793.
41. Ow,D.W., Jacobs,J.D., Howell,S.H. (1987) Proc. Natl. Acad. Sci. USA, **84**,4870–4874.
42. Fang,R.-X., Nagy,F., Sivasubramaniam,S., Chua,N.-H. (1989) The Plant Cell, **1**,141–150.
43. Franck,A., Guilley,H., Jonard,G., Richards,K., Hirth,L. (1980) Cell, **21**,285–294.
44. Dudley,R.K., Odell,J.T., Howell,S.H. (1982) Virology, **117**,19–28.
45. Guilley,H., Dudley,R.K., Jonard,G., Balazs,E., Richards,K.E. (1982) Cell, **30**,763–773.
46. Condit,C. and Meagher,R.B. (1983) J. Molec. Appl. Gen., **2**,301–314.
47. Olszewski,N., Hagen,G., Guilfoyle,T.J. (1982) Cell, **29**,395–402.
48. Yamazaki,K-i. and Imamoto, F. (1987) Mol. Gen. Genet., **209**,445–452.
49. Horikoshi,M., Wang,C.K., Fujii,H., Cromlish,J.A., Weil,P.A., Roeder,R.G. (1989) Nature, **341**,299–303.
50. Miyamoto,N.G., Moncollin,V., Wintzerith,M., Hen,R., Egly,J.M., Chambon,P. (1984) Nucleic Acids Res., **12**,8779–8799.
51. Miyamoto,N.G., Moncollin,V., Egly,J.M., Chambon,P. (1985) The EMBO J., **4**, 3563–3570
52. Sawadogo,M. and Roeder,R.G. (1985) Cell, **43**,165–175.
53. Carthew,R.W., Chodesh,L.A., Sharp,P.A. (1985) Cell, **43**,439–448.
54. Carcamo,J., Lobos,S., Merino,A., Buckbinder,L., Weinmann,R., Natarajan,V., Reinberg,D. (1988) J. Biol. Chem., **264**,7704–7714.
55. Ackerman,S. and Furth,J.J. (1979) Biochemistry, **15**,3243–3248.
56. Sawadogo, M. (1988) J. Biol. Chem., **263**,11994–12001
57. Jove, R. and Manley,J.L. (1984) J. Biol. Chem., **259**,8513–8521.
58. Benfey,P.N., Ren,L., Chua,N.-H. (1989) The EMBO J., **8(8)**,2195–2202.
59. Langridge,P. and Feix,G. (1983) Cell, **34**,1015–1022.
60. Boston,R. and Larkins,B.A. (1986) Plant Molec. Biology, **7**,71–79.
61. Langridge,P., Eibel,H., Brown, J.W.S., Feix,G. (1984) The EMBO J., **3**,2467–2471.
62. Coraggio,I., Compagno,C., Martegani,E., Ranzi,B.M., Sala,E., Alberghina,L., Viotti,A. (1986) The EMBO J., **5**,459–465.
63. Dennis,E. and Berg,P. (1985) Nucleic Acids Res., **13**,7945–7957.
64. Ballas,N., Broido,S., Soreq,H., Loyter,A. (1989) Nucl. Acids Res., **13**,7891–7903.
65. Briggs,M.R., Kadonaga,J.T., Bell,S.P., Tjian,R. (1986) Science, **234**,47–52.
66. Sawadogo,M., Van Dyke,M.W., Gregor,P.D., Roeder,R.G. (1988) J. Biol. Chem., **263**,11985–11993.
67. Hager,D.A. and Burgess,R.R. (1980) Anal. Bioch., **109**,76–86.
68. Cooke,R. and Penon,P. (1990) Plant Molec. Biology, **14**,391–405.
69. Yu,X.-B., Burke,C., Zhang,J., Marchitelli,L., Davis,E.A., Ackerman,S. (1990) Bioch. Biophy. Res. Comm., in press.